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Gas-Liquid Chromatographic Analysis of Fatty Acid Methyl Esters of *Aeromonas hydrophila*, *Aeromonas sobria*, and *Aeromonas caviae*

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Clinical isolates of *Aeromonas hydrophila*, *A. sobria*, and *A. caviae* whose fatty acid content had been analyzed by gas-liquid chromatography (GLC) displayed the following qualitatively similar GLC profiles: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 16:1, 18:1, a-15:0, a-17:0, and 3-OH 14:0. The 16:0/17:0 area-percentage ratio separated the clinical aeromonads in accordance with their species designations. Aeromonads treated with subminimal inhibitory concentrations of the antibiotic cerulenin displayed the following altered qualitative fatty acid GLC profiles: 12:0, 14:0, 16:0, 18:0, 16:1, 18:1, and 3-OH 14:0. Cerulenin-treated cells failed to reproducibly display detectable levels of all odd-numbered-carbon-chain-length fatty acids observed in untreated cells. Cerulenin-treated cells also exhibited overall increases in 14:0 and 3-OH 14:0 and a decrease in total unsaturated fatty acid content.

The genus *Aeromonas* has received a great deal of attention in recent years, not only because of its emergence as a source of pathogens of ever-increasing clinical prevalence but also because of its uncertain taxonomic status. Although presently classified in the family *Vibrionaceae* (31), Colwell et al. (5) recently proposed the elevation of the present genus *Aeromonas* to the family level (*Aeromonadaceae*) on the basis of molecular genetic evidence, including 16S rRNA catalog, 5S rRNA sequence, and rRNA-DNA hybridization data. With respect to the three species of motile aeromonads, namely, *A. hydrophila*, *A. sobria*, and *A. caviae*, the biochemical differentiation scheme of Popoff and Veron has received support from DNA-DNA hybridization studies (26), numerical taxonomy data (16), and core oligosaccharide analysis (28).

Fatty acid methyl ester (FAME) analyses by gas-liquid chromatography (GLC) have been extensively employed in microbiological identification as either primary or adjunct parameters in the routine biochemical testing of many medically important gram-negative bacteria (6–11, 17–23, 29). *Aeromonas* spp., however, have received limited attention in this regard (4, 14). This study was undertaken to determine the total cellular fatty acid composition of 12 strains of clinically derived motile aeromonads (4 strains each of *A. hydrophila*, *A. sobria*, and *A. caviae*) and to assess the utility of GLC as a tool for their differentiation. We also report here on the effect of subminimal inhibitory concentrations of the fatty acid synthesis inhibitor cerulenin on total cellular fatty acid content.

MATERIALS AND METHODS

Cultures. Clinical isolates were obtained from Edward Bottone, The Mount Sinai Hospital, New York, N.Y. A total of 12 strains were studied, including 4 *A. hydrophila*, 4 *A. sobria*, and 4 *A. caviae* strains which had previously been identified by the classification system of Popoff and Veron (25). Cultures for untreated cell analysis were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% defibrinated sheep blood (Hazelton Research Products, Denver, Pa.). Plates were incubated at 37°C for 24 h before either cerulenin susceptibility testing or harvesting for fatty acid analysis. Cultures for cerulenin-treated cell analysis were grown on plates as described above with the addition of a predetermined concentration of cerulenin (Sigma Chemical Co., St. Louis, Mo.).

Fatty acid analysis. Each strain was analyzed on three separate occasions by the method of Moss et al. (21) for saponification, methylation, and FAME extraction. To ensure that all bound hydroxy acids were liberated, we used the procedure of Lambert and Moss (15).

All FAME samples were analyzed by GLC with a fused-silica wide-bore capillary column (30 m by 0.75 mm [inside diameter]) coated with 1.0-μm bonded methyl silicone (SPB-1) as the stationary phase (Supelco, Inc., Bellefonte, Pa.). The column was installed in a gas chromatograph (Sigma 3B; The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a flame ionization detector. This packed-column instrument was adapted to perform wide-bore capillary analyses with a wide-bore capillary conversion kit (Supelco). For FAME analyses, the column was temperature programmed from 150 to 240°C at 6°C/min and was maintained at 240°C for 7 min. The injector temperature was 250°C, and the detector temperature was 260°C. The carrier gas was nitrogen at a flow rate of approximately 5 ml/min at the injection port. We used a makeup gas kit (Supelco) to provide a booster flow of nitrogen at approximately 48 ml/min to the detector end of the column to minimize sample band spreading before passage through the flame ionization detector. Sample sizes varied from 0.5 to 0.75 μl. Quantitation of all chromatographic profiles was performed with an integrator (model 3390A; Hewlett-Packard Co., Avondale, Pa.).

Tentative identification of FAME peaks was achieved by comparison of sample retention times with the retention times of commercially prepared FAME standard mixes and individual hydroxy FAMEs (Supelco). Final identification was achieved through trifluoroacetylation of hydroxy acids (18), hydrogenation of unsaturated acids (18), and bromination (2).

Cerulenin susceptibility testing. Frozen broth microdilution

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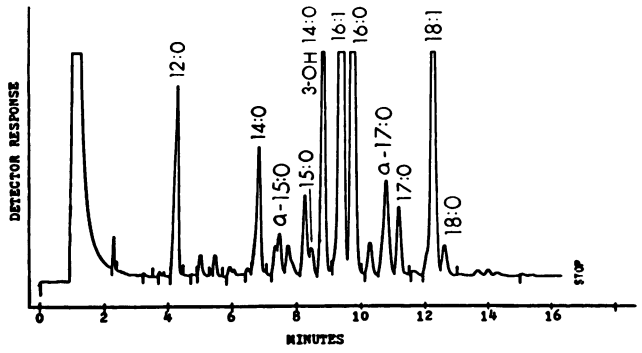

FIG. 1. Gas chromatogram of *A. hydrophila* isolate 4. Analysis was conducted with a 30-m fused-silica wide-bore capillary column coated with 1-μm bonded methyl silicone.

tests (24) were used to determine the MIC range of cerulenin for each strain. A total of 40 MIC readings (10 readings per day for 4 successive days) established the modal MIC for a strain (defined as the MIC obtained in more than 60% of total readings). From this information was derived the specific subminimal inhibitory concentration of cerulenin (defined as 1 twofold serial dilution below the modal MIC) to which the strain was exposed prior to extraction, methylation, and GLC analysis of fatty acids produced under antimicrobial stress conditions.

Statistical analyses. Analyses of variance were performed by using the Student *t* test. When the means of two or more small samples were being compared, the statistical equality of the variances of those samples was tested by the variance ratio test, also known as the *F* test, prior to *t*-test analysis (1).

RESULTS

Under normal growth conditions, each of the untreated aeromonads studied displayed a GLC profile for FAME species which was qualitatively similar to that obtained for *A. hydrophila* isolate 4 (Fig. 1). Each strain contained the following FAME species: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 16:1, 18:1, a-15:0, a-17:0, and 3-OH 14:0. Although qualitative differences in fatty acid composition were not observed, the aeromonads did exhibit quantitative differences in the relative amounts of individual fatty acids. The most abundant fatty acids present in untreated cells were 16:0, 16:1, and 18:1, which collectively accounted for approximately 60% of total peak area for each isolate.

The 16:0/17:0 area-percentage ratio clearly sorted the 12 clinically derived untreated aeromonads into three distinct groups which corresponded to their species designations (Table 1). The 16:0/17:0 area-percentage ratio of *A. hydrophila* strains ranged from 5.58 to 10.2, with ranges of 14.34 to 28.49 and 31.74 to 57.99 for *A. sobria* and *A. caviae*, respectively. The differences between the mean ratios for these taxonomic clusters were determined to be statistically significant ($P < 0.001$).

Cerulenin-treated aeromonads displayed GLC profiles qualitatively similar to that obtained for *A. hydrophila* isolate 4, which contained the following FAME species: 12:0, 14:0, 16:0, 18:0, 16:1, 18:1, and 3-OH 14:0 (Fig. 2). The FAME species 14:0, 16:0, and 16:1 were the most abundant, collectively accounting for approximately 60% of the total peak area for each isolate. Exposure to maximum tolerable levels of cerulenin produced a uniform change in FAME

TABLE 1. Differentiation of aeromonads based on the 16:0/17:0 FAME area-percentage ratio

Isolate	FAME area-percentage ratio	
	16:0/17:0	Mean
A. hydrophila		8.59
4	9.48	
11	5.58	
19	10.20	
25	9.10	
A. sobria		22.68
14	28.49	
24	26.06	
28	14.34	
30	21.82	
A. caviae		42.28
5	32.08	
23	31.74	
34	47.29	
36	57.99	

content for all aeromonads studied. It was observed that all FAME species of odd-numbered-carbon-chain length originally detected in each untreated isolate were no longer present at detectable levels in their cerulenin-treated counterparts. Cerulenin-induced stress on the aeromonads caused the disappearance of FAME species 15:0, 17:0, a-15:0, and a-17:0. Only FAME species of even-numbered-carbon-chain length persisted in cerulenin-treated aeromonads.

Among even-numbered-carbon-chain-length FAME species, myristic acid (14:0) demonstrated the most drastic change in content between untreated and cerulenin-treated aeromonads. Myristic acid, a relatively minor constituent of untreated aeromonads with an area-percentage ranging from 1.69 to 5.24, markedly increased in relative area-percentage under conditions of cerulenin inhibition for all strains studied (Table 2). The net area-percentage increase of 14:0 content ranged from 15.78% for *A. caviae* isolate 5 to 25.12% for *A. sobria* isolate 24. The average myristic acid increase for the 12 test strains was 20.27% of total peak area.

All of the aeromonads examined exhibited only one hydroxy FAME species, namely, 3-OH 14:0, at a level greater than 1% on a reproducible basis. Of 12 aeromonads, 11

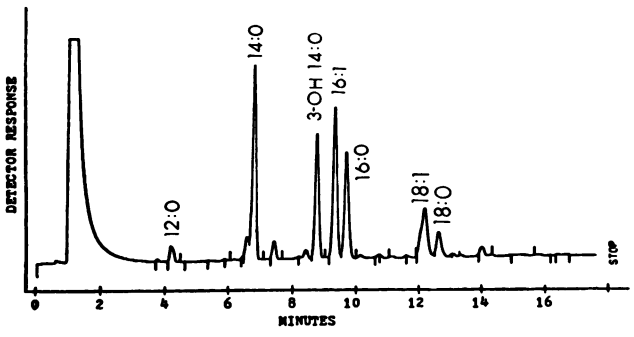

FIG. 2. Gas chromatogram of cerulenin-treated *A. hydrophila* isolate 4. Analysis was conducted with a 30-m fused-silica wide-bore capillary column coated with 1-μm bonded methyl silicone.

TABLE 2. Change in myristic acid content of aeromonads treated with cerulenin

Isolate	Myristic acid content (%)		
	Untreated cells	Cerulenin-treated cells	Net change
A. hydrophila			
4	4.05	21.23	+17.18
11	4.01	23.34	+19.33
19	4.05	25.48	+21.43
25	4.26	25.08	+20.82
A. sobria			
14	5.24	25.68	+20.44
24	1.69	26.81	+25.12
28	4.07	27.99	+23.92
30	2.51	26.42	+23.91
A. caviae			
5	3.46	19.24	+15.78
23	3.24	21.41	+18.17
34	2.38	21.86	+19.48
36	3.25	20.92	+17.67

displayed higher concentrations of 3-OH 14:0 when treated with cerulenin than when untreated (Table 3). *A. sobria* isolate 24 was the sole isolate which exhibited a decrease in 3-OH 14:0. The net increase of 3-OH 14:0 under cerulenin stress ranged from 3.28% for *A. caviae* isolate 34 to 8.29% for *A. caviae* isolate 36, with an average increase for the 11 strains of 6.3%.

Finally, cerulenin exposure led to an overall decrease in the total unsaturated fatty acid content of 11 of the aeromonads studied. Only *A. caviae* isolate 36, with a modest 3.04% increase, demonstrated greater total unsaturated fatty acid content when treated with cerulenin (Table 4). The net unsaturated fatty acid decrease under cerulenin stress ranged from 6.14% for *A. caviae* isolate 5 to 20.01% for *A. sobria* isolate 30. The average unsaturated fatty acid decrease for the 11 strains was 11.92% of total peak area.

TABLE 3. Change in 3-OH 14:0 content of aeromonads treated with cerulenin

Isolate	3-OH 14:0 content (%)		
	Untreated cells	Cerulenin-treated cells	Net change
A. hydrophila			
4	8.57	13.74	+5.17
11	8.00	15.99	+7.99
19	8.41	14.09	+5.68
25	7.77	14.33	+6.56
A. sobria			
14	7.14	14.94	+7.80
24	17.26	13.14	−4.12
28	7.98	12.89	+4.91
30	5.61	13.47	+7.86
A. caviae			
5	8.97	14.34	+5.37
23	9.94	16.31	+6.37
34	8.29	11.57	+3.28
36	6.83	15.12	+8.29

TABLE 4. Change in total unsaturated fatty acid content of aeromonads treated with cerulenin

Isolate	Total unsaturated fatty acid content (%)		
	Untreated cells	Cerulenin-treated cells	Net change
A. hydrophila			
4	44.26	31.34	−12.92
11	38.60	31.29	−7.31
19	40.70	31.30	−9.40
25	39.13	26.79	−12.34
A. sobria			
14	37.76	25.69	−12.07
24	31.55	21.52	−10.03
28	38.45	18.95	−19.50
30	41.01	21.00	−20.01
A. caviae			
5	42.68	36.54	−6.14
23	42.30	33.63	−8.67
34	36.66	23.95	−12.71
36	31.66	34.70	+3.04

DISCUSSION

In this investigation, the total cellular fatty acid compositions of 12 clinically derived strains of motile aeromonads cultured under conditions normally used in the clinical microbiology laboratory have been characterized. These strains displayed qualitatively similar FAME profiles when analyzed by GLC. The motile aeromonads lacked (i) the cyclopropane fatty acids normally found in genera of the family *Enterobacteriaceae* (14), (ii) some of the hydroxy fatty acids such as 2-OH 14:0, 3-OH 13:0, and iso-3-OH 15:0 found in the family *Vibrionaceae* (14), and (iii) the large variety of branched-chain acids characteristic of the family *Legionellaceae* and many gram-positive bacteria (17, 23). The major fatty acid constituents of the motile aeromonads (16:0, 16:1, and 18:1) bore similarity to those of other members of the family *Vibrionaceae* previously documented (14), although extreme care must be taken in making fatty acid content comparisons between investigations in which culture conditions are different, since the availability of fatty acid precursors such as acetyl coenzyme A (acetyl-CoA), propionyl-CoA, branched-chain amino acids, and end products or by-products of fatty acid oxidation can markedly influence cellular fatty acid composition (13, 23).

The area-percentage ratio has been used in the past as a means of expressing quantitative differences among chromatographic profiles (14, 21). The 16:0/17:0 area-percentage ratio differentiated with statistical significance the clinically derived strains into the three presently recognized motile aeromonad species, namely, *A. hydrophila*, *A. sobria*, and *A. caviae*. Further investigation with larger numbers of clinical *Aeromonas* strains will be required, however, to more firmly establish the significance of this characteristic.

The exposure of each test strain to the fatty acid synthesis inhibitor cerulenin led to the determination of the fatty acid profile of each strain under the maximal stress conditions imposed by this antimicrobial agent which still permitted cell growth and replication. Cerulenin exerts its effect by binding irreversibly with beta-ketoacyl-acyl carrier protein (ACP) synthetase of the fatty acid synthetase complex. The highly specific nature of the covalent interaction between cerulenin and beta-ketoacyl-ACP synthetase has been demonstrated

through the observation that a linear relationship exists between the amount of cerulenin bound to the enzyme and the extent of enzyme inhibition (12, 30).

As previously mentioned, the aeromonads examined in this study displayed an overall decrease in unsaturated fatty acid content under conditions of cerulenin inhibition. The total content of saturated fatty acids in cerulenin-treated aeromonads, on the other hand, increased over that of untreated cells. This finding suggests that the motile aeromonads might also contain two beta-ketoacyl-ACP synthetases which exhibit differential cerulenin susceptibilities in much the same fashion as do those of *Escherichia coli*.

In *E. coli*, the presence of two beta-ketoacyl-ACP synthetases (designated I and II) has been demonstrated (3). Mutants deficient in beta-ketoacyl-ACP synthetase I lack the capability to synthesize unsaturated fatty acids, while mutants deficient in beta-ketoacyl-ACP synthetase II produce only minimal amounts of saturated fatty acids. Differential sensitivity of the two enzymes to cerulenin was also observed, with synthetase I exhibiting far greater sensitivity to the antibiotic than did synthetase II.

Under conditions of cerulenin exposure, all of the aeromonads examined in this study displayed far greater quantities of myristic acid (14:0) than did untreated cells. Similar increases in myristic acid content in cerulenin-treated cells have previously been documented for both *E. coli* and *Proteus mirabilis* (3, 27). Pulse-labeling studies conducted with *E. coli* have indicated that the increase in myristic acid was due to an increased amount of saturated fatty acid synthesis and was not a result of an inhibition of elongation of 14:0 to 16:0. The preferential inhibition of unsaturated fatty acid synthesis in *E. coli* (through preferential inhibition of beta-ketoacyl-ACP synthetase I) apparently resulted in an accumulation of acyl intermediates which could be utilized for saturated fatty acid synthesis (3).

Finally, the absence of detectable levels of odd-numbered-carbon-chain-length fatty acids in cerulenin-treated aeromonads fosters speculation regarding this phenomenon. One possible explanation is that there was an overall lack of available propionyl-CoA in these cells, a condition which would preclude the synthesis of odd-numbered carbon chains. A second possible explanation is that beta-ketoacyl-ACP synthetase II in aeromonads may exist in more than one conformation. One conformation of the enzyme might display greater affinity for acetyl-CoA, while the other displays greater affinity for propionyl-CoA. If cerulenin exhibited preferential affinity for the conformation which binds propionyl-CoA, then the synthesis of odd-numbered carbon chains would significantly decrease.

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